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Therapeutic potential of CAMPATH-1H in skeletal tumours

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For Peer Review

Abstract

Aim: CD52 is a GPI-anchored glycoprotein that is expressed abundantly on all lymphocytes, monocytes, macrophages, eosinophils and in the male genital tract. To date, the physiological role of CD52 on lymphocytes has not been elucidated. However, an antibody directed to CD52 called CAMPATH-1H has been shown to be capable of depleting lymphocytes. Tissue and cell lines of non-neoplastic bone, cartilage and skeletal tumours were analysed for CD52 expression. **Methods and results:** We detected the expression of the CD52 mRNA and protein both *in vivo* and *in vitro*. The malignant tumours showed a higher CD52 expression compared to the benign tumours, suggesting a role in the development and progression of bone tumours. Interestingly, immunohistochemistry and flow cytometry revealed that CD52 was not only expressed on the surface of the tumour cells, but also in the cytoplasm. Our results obtained in osteosarcoma cells show that CAMPATH-1H leads to a complement-independent reduction of viable cells. **Conclusion:** CD52 is expressed in a variety of bone tumours and the *in vitro* studies presented herein suggest that CAMPATH-1H treatment might have therapeutic potential for osteosarcoma patients with poor clinical prognosis.

Introduction

CD52 is a glycosylphosphatidylinositol (GPI)-anchored antigen, also called the CAMPATH-1H antigen. It is abundantly expressed on normal and malignant lymphocytes, such as cells from most B- and T-cell malignancies and in a minority of myeloid leukaemias on monocytes, macrophages and eosinophils^{1, 2}. In addition, CD52 can be found in the epithelial cells of the distal epididymis and the vas deferens and is acquired by sperm cells during their passage through the genital tract³. The structure of the CD52 protein is unusual. It is a very small, heavily glycosylated antigen with glycolipid-like properties. The sperm CD52 protein differs from the lymphocyte CD52 protein in its carbohydrate structure⁴. The physiological role of CD52 on lymphocytes is unclear. The high density of CD52 and the close proximity of the antibody binding site to the cell surface make it a particularly good target for therapeutic options⁵. These properties may contribute to the efficacy of the therapeutically used CD52 antibody, also called CAMPATH-1H (Alemtuzumab). CAMPATH-1H is capable of complement-activation and antibody-mediated cellular cytotoxicity leading to a depletion of lymphocytes^{6, 7}. *Stanglmaier et al.* also suggested a caspase-independent form of apoptosis that may be induced by CAMPATH-1H in patients with chronic lymphocytic leukaemia⁸. At present, CD52-specific antibodies are used to treat lympho-proliferative disorders like B-cell chronic lymphocytic leukaemia, to deplete lymphocytes in bone marrow transplants, in the therapy of transplant rejection, to control graft versus host disease and, most recently, to treat Behcet's disease and multiple sclerosis⁹⁻¹⁴.

By microarray analysis, we found that *CD52* mRNA is significantly overexpressed in giant cell tumours of the bone¹⁵. Further experiments revealed a significant upregulation of *CD52* mRNA in chondrosarcoma and osteosarcoma tissue samples, suggesting a previously unknown deregulation of CD52 in bone malignancies.

The current study investigates levels of CD52 expression in skeletal tumours, especially in giant cell tumours, osteosarcoma and chondrosarcoma tissue and cell lines and their equivalent non-tumorous counterparts. The results imply a role for CD52 in the development and the progression of bone tumours. Furthermore, results obtained in osteosarcoma cells show that CAMPATH-1H leads to a reduction of viable cells. Therefore, CAMPATH-1H might have therapeutic potential for osteosarcoma patients with poor clinical prognosis.

Material and Methods

Tissue studies

Patients and tissues

Tumour tissue samples were obtained from the Institute of Pathology, Charité Universitätsmedizin Berlin, Berlin, Germany and the Department of Pathology, University of Cologne, Cologne, Germany. Patient characteristics are described in Table 1. The tissue specimens were fixed in 4% neutral buffered formaldehyde, and bone-containing tissue was EDTA decalcified and embedded in paraffin. For each case, hematoxylin and eosin (H&E)-stained slides were carefully reviewed and the diagnosis of the tumour was confirmed according to the WHO criteria by two different pathologists. Tissue microarrays of bone (n=4 foetal and n=6 adult bone samples, each in duplicates; Cat. No. 4011211), cartilage (n=4 embryonal, n=6 foetal and n=10 adult cartilage samples, each in duplicates; Cat. No. 4011223) and skeletal tumours (n=3 fibrosarcomas, n=2 osteosarcomas, n=3 chondrosarcomas, n=1 solitary bone cyst, n=4 aneurysmal bone cysts, n=1 fibrous dysplasia, n=5 chondromas, n=5 osteomas, n=4 osteochondromas, each in duplicates; Cat. No. 4132212) were obtained from Provitro GmbH, Berlin, Germany. Written informed consent was obtained from each patient.

Immunohistochemistry

Immunohistochemical analysis of CD52 was performed on 26 giant cell tumours (n=18 primary tumours, n=8 relapses), 14 osteosarcomas (n=7 conventional, n=7 metastases) and 6 chondrosarcomas on paraffin-embedded microsections. Three tissue microarrays of cartilage, bone and skeletal tumours were stained for CD52 expression. Tissues were cut into 1-3 µm sections and deparaffinised through serial dilutions of alcohol. Retrieval of the CD52 antigen was performed by incubation with proteinase K (DAKO) and 0.05% trypsin (Zymed). Anti-CD52 antibody (1:40 rat anti-human, Serotec), secondary antibody (1:40 rabbit anti-rat DAKO) and the APAAP complex (1:100 rat DAKO) were incubated for 30 min, 10 min and 10 min at room temperature, respectively. Fuchsin⁺ (DAKO) was used as the chromogen. Nuclear counter staining was performed with haemalum. Negative control sections were not incubated with the primary antibody and in no case showed evidence of staining with colour reactions under microscopic examination.

The staining of all cases was semi-quantitatively scored by two different observers using the following method modified from *Remmele and Stegner*¹⁶: 1. A score to measure the percentage of positively stained cells (PP) was performed as follows: 0 = no staining, 1 = 1-25%, 2 = 26-50%, 3 = 51-75%, 4 = 76-100%. 2. A staining intensity score (SI) was established to measure the intensity of positively stained cells: 0 = no expression, 1 = weak expression, 2 = moderate expression, 3 = strong expression. The combined immunoreactivity score (IRS) value was calculated through PPxSI with a maximum value of 12. Statistical analyses were performed executing the Mann-Whitney-U-Test using SPSS 13.0 software. Differences were considered significant at p<0.05.

In vitro studies

Cells and cell culture

Primary cultures of osteoblasts (HOBc) and chondrocytes were purchased from Provitro GmbH, Berlin, Germany and cultured in special osteoblast growth medium (PromoCell, Heidelberg, Germany) or RPMI1640 media (Biochrom) supplemented with 10% foetal calf serum and 1% penicillin/streptomycin, respectively. The cell line Hut78 was obtained from the ATCC (American Type Culture Collection, United Kingdom) and cultured in RPMI1640 media (Biochrom) supplemented with 10% foetal calf serum and 1% penicillin/streptomycin. The osteosarcoma cell line MNNG/HOS was a gift from Prof. Dr. G. Gaedicke, Clinic for General Paediatrics and Experimental Oncology Charitè Universitätsmedizin Berlin, Berlin, Germany. The cells were maintained in RPMI1640 media (Biochrom) supplemented with 10% foetal calf serum and 1% penicillin/streptomycin. The chondrosarcoma cell line AD was a gift from Dr. T. Aigner, Institute of Pathology, Friedrich-Alexander University Erlangen, Erlangen, Germany. For the establishment of a human giant cell tumour primary culture, resection tissue (male, 28 years) was minced in dispase-collagenase with PBS (phosphate buffered saline) and incubated for 20 min at 37°C. After that the cells were resuspended in Leibowitz L15 medium (Lonza) and, together with small tissue fragments, were then transferred to 75-cm² flasks for subsequent culturing. Half of the medium was changed on the second day. All cells were incubated in a humidified atmosphere of 5% CO₂ in air at 37°C.

Flow cytometry

Direct fluorescein isothiocyanate (FITC)-conjugated rat anti-human CD52 antibody (1:10 Serotec) and FITC-conjugated IgG (1:50 Pharmingen) were used for flow

analyses of Hut78, HOBc, MNNG/HOS, chondrocytes, AD and giant cell tumour cell lines. The flow cytometry was performed by staining the cells with the FITC-conjugated antibody in PBS containing 1% BSA (bovine serum albumine) and 0.2% Saponin for intracellular staining and in PBS containing 1% BSA for membranous staining for 30 min. Prior to the intracellular staining the cells were treated with 8% paraformaldehyde for 15 min at 37°C for fixation. Cells were analysed using a FACScan (BectonDickinson).

Proliferation assay

MNNG/HOS cells were plated in 96-well-plates and treated with 10-1,000 µg/ml CAMPATH-1H (Schering) for up to 96 h. For complement-mediated cytotoxicity, 10% human complement was added to the media. For the control, the complement was heat-inactivated for 1 h at 56°C. Proliferation of cells was measured using the MTT cell proliferation kit from Roche.

Immunoblot

Protein extracts of MNNG/HOS cells were prepared by incubation with sodium dodecylsulfate (SDS)-cell lysis buffer (10% SDS, 1M Tris-HCl pH 7.5, EDTA 0.5 M pH 8). Reagents for SDS-PAGE and Western blotting were obtained from Bio-Rad Laboratories (Richmond, CA). Electrophoresis was performed and the proteins were transferred onto nitrocellulose membranes (Schleicher & Schüll). Unbound protein sites were blocked with 5% milk powder in Tris-buffered saline containing 0.01% Tween-20 (TBST). Thereafter, specific proteins were detected by incubation with mouse anti-human PARP (1:500 BD) and mouse anti-human actin (1:5,000 Chemicon) antibodies

overnight at 4°C followed by HRP-conjugated rabbit anti-mouse (1:5,000 Dianova) secondary antibody. Blots were developed using the ECL system from Amersham.

Results

Tissue studies

Increased CD52 expression in bone malignancies

RNA derived from eight osteosarcoma samples (n=4 conventional, n=4 metastases) and from the human osteoblast primary culture HOBc was analysed using Affymetrix HG U133A microarrays (GEO accession number GSE14359; Guenther et al. submitted). A significantly higher expression of *CD52* was found in osteosarcoma metastases as compared to conventional osteosarcoma (p=0.000619). *CD52* mRNA levels were below background in HOBc cells and the conventional osteosarcomas, except for in the OS18 cell line (relative expression level of 1,022 and 1,336). In the microarray analysis of primary and recurrent giant cell tumours of bone, *CD52* mRNA was lower in recurrent as compared to primary giant cell tumours¹⁵.

For the validation of microarray data, 14 osteosarcoma tissue samples (n=7 conventional and n=7 lung metastases) and 26 giant cell tumour tissue samples (n=18 primary, n=8 recurrence) were stained for CD52. As a control, a tissue microarray of non-neoplastic bone samples containing 4 foetal and 6 adult samples was used. High levels of CD52 protein were seen in foetal bone (Fig. 1A), while adult bone had lower CD52 levels (Fig. 1B). Interestingly, the osteoblasts in foetal bone samples showed the strongest CD52 staining, while the osteoblasts in adult bone were CD52 negative or showed only weak expression of CD52. We also found a largely cytoplasmic localisation, with only rare cases having a nuclear expression pattern of CD52 protein. The osteocytes were CD52 positive in foetal and adult bone (Fig. 1A, B). The primary

osteoblast cells HOBc did not express CD52 protein, consistent with our Affymetrix mRNA data (Fig. 1C). All osteosarcoma samples showed a strong immunoreactivity for the CD52 antigen (IRS 6.1), but no significant differences could be observed between the conventional osteosarcomas and the metastases ($p=0.515$). The latter showed generally a lower CD52 expression (IRS=5.6) as compared to the conventional osteosarcomas (IRS=6.6). In most cases, the CD52 staining was cytoplasmic, but some nuclear localisation could also be observed (Fig. 1D).

CD52 protein was detected in 18/18 primary giant cell tumours and 8/8 recurrence samples. The macrophage-like cells and the multinucleated giant cells showed on average a moderate cytoplasmic staining intensity (Fig. 1E).

High expression of CD52 in chondrosarcoma and cartilage

CD52 protein expression was also tested in non-neoplastic and neoplastic cartilage samples. The non-neoplastic tissue microarray included 4 embryonal, 6 foetal and 10 adult cartilage tissue samples, and the neoplastic tissue array had 6 chondrosarcoma tissue samples. Non-neoplastic cartilage was CD52 negative in all embryonal (Fig. 2A) and in 4/5 foetal cartilage samples (Fig. 2B). Only one foetal cartilage tissue had low CD52 expression. In contrast, CD52 expression could be observed in chondrocytes of adult cartilage by immunohistochemistry (Fig. 2C). Furthermore, a primary chondrocyte culture showed a low cytoplasmic CD52 expression (Fig. 2D). The chondrosarcoma tissue samples had the highest IRS (IRS 7.3) compared to osteosarcomas (IRS 6.1) and giant cell tumours (6.2). In total, 76-100% of the chondrosarcoma cells were positive (PP=3.8) with an average staining intensity of 2.1. The grade 2 chondrosarcomas showed a moderate cytoplasmic expression of CD52, whereas in the grade 3 samples a strong cytoplasmic CD52 staining could be observed (Fig. 2E). Because of the small

sample number, no statistically significant correlation between protein intensity and tumour grade could be shown.

CD52 expression is rare in skeletal tumours

In addition to osteosarcoma, chondrosarcoma and giant cell tumours, benign bone- and cartilage-related, malignant fibrous and skeletal tumours with undefined origin (fibrous dysplasia and aneurysmal bone cyst) were analysed for CD52 expression. Results from immunohistochemical evaluation of all skeletal tumours are shown in Table 2. Cytoplasmic and nuclear CD52 expression could be detected in 4/5 benign chondromas (Fig. 3A), 2/3 benign osteochondromas (Fig. 3B), 3/3 malignant fibrosarcomas (Fig. 3C), 3/3 benign aneurysmal bone cysts (Fig. 3D) and 5/5 benign osteomas (Fig. 3E). Fibrous dysplasia stained weakly and CD52 protein was only detected in the stroma (Fig. 3F).

***In vitro* studies**

Membranous and intracellular localisation of CD52

Immunohistochemical staining suggested a dual localisation of CD52 both on the surface of the cells, as expected for GPI-anchored glycoproteins, but also in the cytoplasm. To analyse the intra- and extracellular expression of CD52, flow cytometric analysis was performed using the CD52 FITC-conjugated antibody in osteosarcoma and chondrosarcoma cell lines, giant cell tumour and chondrocyte and osteoblast primary cultures (Table 3, Fig. 4). The human T-cell lymphoma cell line Hut78 was included as a positive control due to known expression of CD52¹⁷.

Staining of Hut78 for membranous and intracellular CD52 revealed 32% and 93% positive cells, respectively (Fig. 4A). In MNNG/HOS osteosarcoma cells, 10% of cells showed a membranous staining, while 69% showed an intracellular staining (Fig.

4C). The chondrosarcoma cell line AD contained 74% positive cells, the highest proportion of CD52 positive cells (Fig. 4E). The highest membranous expression could be detected in the giant cell tumour primary culture, with 20% positive stained cells (Fig. 4F).

CAMPATH-1H inhibits growth of osteosarcoma cells

The therapeutic CD52 antibody CAMPATH-1H was shown to deplete lymphocytes through antibody-dependent cytotoxicity or complement-dependent cytotoxicity. To study the effect of the CAMPATH-1H antibody on bone tumours, we incubated the human osteosarcoma cell line MNNG/HOS with 50 µg/ml or 100 µg/ml CAMPATH-1H and assayed for cell growth.

Using 50 µg/ml and 100 µg/ml CAMPATH-1H in media containing 10% human complement, a significant reduction of proliferating MNNG/HOS cells was observed when compared to cells incubated with inactive complement (50 µg/ml $p=0.026$, 100 µg/ml $p=0.021$; Fig. 5A). To test the specificity of CAMPATH-1H, the same assay was performed in the absence of human complement. This assay demonstrated a dose- (Fig. 5B) and time-dependent (Fig. 5C) reduction of viable MNNG/HOS cells compared to untreated control cells. To determine whether CAMPATH-1H induces apoptosis, we performed Western blot analysis for cleaved PARP. Apoptosis leads to cleavage of the 116 kDa native PARP protein to an 85 kDa fragment. In MNNG/HOS CAMPATH-1H treated cells, no cleaved PARP product was observed, suggesting an apoptosis-independent mechanism of cell growth inhibition (Fig. 5D).

Discussion

CAMPATH-1H, also known as Alemtuzumab, belongs to the CAMPATH-family of antibodies that recognises CD52. The CD52 antigen is highly expressed on lymphocytes, monocytes, eosinophils and in the male reproductive tract³. The function of CD52 is still unknown. Nevertheless, the CAMPATH-1H antibody is effective in the treatment of chronic lymphocytic leukaemia¹⁸. Our current work shows for the first time that CD52 is also expressed in non-neoplastic and neoplastic bone- and cartilage-related mesenchymal cells, suggesting a role in bone or cartilage development and progression.

We found high CD52 levels in malignant tumours like chondrosarcoma and osteosarcoma but lower expression in benign and semimalignant tumours. Interestingly, the cartilage-forming tumours showed the highest expression of CD52. A high level of CD52 protein was detected in non-neoplastic cartilage and the benign tumours osteochondroma and chondroma, and an even stronger staining was found in chondrosarcoma. Based on these observations we suggest that CD52 expression in bone tumours correlates with the degree of malignancy. However, due to the small sample number, no statistical correlation between protein intensity and tumour grade could be shown. This observed upregulation of CD52 expression is consistent with reported increased levels of soluble CD52 in patients with chronic lymphatic leukaemia¹⁹.

CD52 was also strongly expressed in foetal reactive bone, while quiescent adult bone cells showed weak or no expression of the CD52 antigen. High expression of CD52 in osteosarcoma suggests the reactivation of an embryonic program in these tumours. One of the best known examples indicating the reactivation of embryonic genes in tumours is the expression of the carcinoembryonic antigen (CEA). CD52 and CEA share several properties; for example, patients with osteosarcoma show an increased serum level of CEA²⁰. Comparable to CEA, soluble CD52 was also found in

the serum of patients with chronic lymphatic leukaemia, indicating that a potential shedding of CD52 might also occur in osteosarcoma cells^{19, 21}. These observations suggest that soluble CD52 may act as a tumour marker for chronic lymphatic leukaemia, because increased CD52 levels correlate with aggressive stages of the disease. Whether CD52 can potentially also be used as a tumour marker in the serum of patients with osteosarcoma has yet to be determined.

Microarray analysis of osteosarcoma tissue suggested a significantly higher *CD52* expression in lung metastases of osteosarcomas as compared to conventional osteosarcomas. In contrast, the microarray analysis of primary and recurrent giant cell tumours of the bone showed a significantly higher expression of CD52 in primary tumours as compared to the recurrent¹⁵. These differences could not be verified using immunohistochemistry. The differential expression of CD52 was based on variable fractions of lymphocytes and bone tumour cells. For example, in the osteosarcoma metastases, more monocytes and macrophages could be detected than in the conventional osteosarcomas (data not shown) leading to an artificially increased *CD52* mRNA level. In the giant cell tumour recurrent tissue, decreased macrophages and lymphocytes were detected as compared to the primary tumours, leading to an artificial decrease in *CD52* expression.

Interestingly, an intracellular expression of CD52 flow cytometry could be detected. In a previous study, the GPI-anchored protein EFNA1 was also shown to be expressed intracellularly, which could be due to abnormalities in GPI-anchor biosynthesis or failure of GPI transport resulting in an intracellular localisation of CD52. Nevertheless, CAMPATH-1H was able to deplete osteosarcoma cells *in vitro* with and without complement. Based on these results, further studies are warranted to

determine whether CAMPATH-1H could be a therapeutic option to treat patients with osteosarcoma tumours.

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Tables

Table 1: Patient samples of tissues used for immunohistochemical analyses.

Tumour		Type		Mean age	Gender
Bone	n=10	foetal	n=4		
		adult	n=6	68.2	n=1 male n=5 female
Osteosarcoma	n=14	conventional	n=7	42.6	n=5 male n=2 female
		metastases	n=7	39.4	n=4 male n=3 female
Cartilage	n=20	embryonal	n=4		
		foetal	n=6		
		adult	n=10	69.2	n=6 male n=4 female
Chondrosarcoma	n=8			48.5	n=7 male n=1 female
GCT	n=26	primary	n=18	39.8	n=9 male n=9 female
		recurrence	n=8	42.9	n=4 male n=4 female
Osteoma	n=5			38.2	n=3 male n=2 female
Osteochondroma	n=3			21.6	n=1 male n=2 female
Chondroma	n=5			65.0	n=5 female

Fibrosarcoma	n=3	48.3	n=3 male
Aneurysmal bone cyst	n=3	24.3	n=2 male n=1 female
Fibrous dysplasia	n=1	60	male

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Table 2: Immunohistochemical analyses of skeletal tumours. CD52-negative and CD52-positive tissue staining and the staining intensity (+: low, ++: medium, +++: strong) was calculated for each sample.

Origin	Tumour	Type	Classification	CD52 expression
Cartilage	Chondroma	Enchondroma	Benign	1/1 ++
		Periosteal	Benign	3/4 +
	Osteochondroma		Benign	2/3 +
	Chondrosarcoma		Malignant	6/6 +++
Bone	Osteoma		Benign	5/5 +
	Osteosarcoma		Malignant	14/14 +++
Fibrous	Fibrosarcoma		Malignant	3/3 +++
Undefined	Fibrous dysplasia		Benign	1/1 +
	Aneurysmal bone cyst		Benign	3/3 +
GCT			Semimalignant	26/26 ++

Table 3: Membranous and intracellular expression of CD52 analyzed by flow cytometry. %: Percentage of CD52 positive cells. GCT: Giant cell tumour primary culture.

	Membranous			Intracellular		
	% IgG	% CD52	% IgG-CD52	% IgG	% CD52	% IgG-CD52
Hut78	2.0	34.2	32.2	2.3	94.9	92.6
HOBc	0.3	0.5	0.2	0.3	0.4	0.1
MNNG/HOS	0.1	9.9	9.8	0.1	69.0	68.9
Chondrocytes	1.7	2.9	1.2	1.2	8.0	6.8
AD	2.0	3.3	1.3	0.7	74.4	73.7
GCT	5.3	25.0	19.7	4.7	54.9	50.2

Figure legends

Figure 1: **A:** CD52 protein expression in human fetal bone. The osteocytes (blue arrow) and the osteoblasts (black arrow) were CD52 positive. **B:** No or weak CD52 staining in human adult bone osteoblasts (black arrow), while a CD52-expression is observed in the osteocytes (blue arrow). **C:** HOBc osteoblast cells are CD52 negative. **D:** CD52-expressing tumor cells in a high grade conventional osteosarcoma. **E:** CD52-expression in giant cell tumors of bone. Most of the cells (including multinuclear giant cells, mononuclear-like cells, fibroblast-like cells and matrix) were CD52-positive, while also negative cells could be detected (arrow). So we excluded general background staining. **F:** Heightened picture of the CD52-negative multinucleated giant cell of E.

Figure 2: Human embryonal cartilage is negative for CD52 (**A**); also human fetal cartilage does not express CD52 (**B**). Adult cartilage expresses the CD52 antigen (**C**). Low CD52-expression in the primary culture of chondrocyte cells (**D**) and a strong CD52-expression in chondrosarcoma tumor cells (**E**).

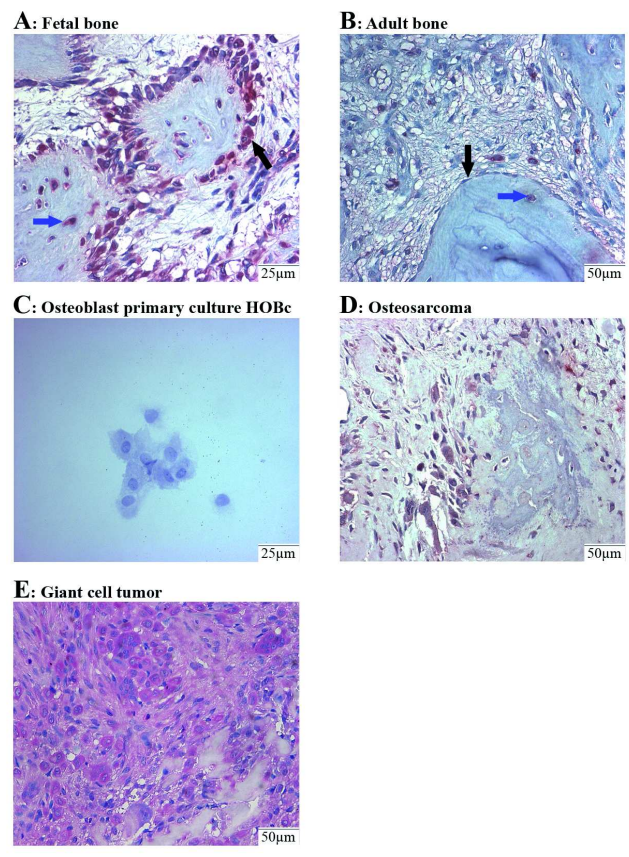
Figure 3: Chondroma (**A**), osteochondroma (**B**), fibrosarcoma (**C**), aneurysmal bone cyst (**D**), osteoma (**E**) and fibrous dysplasia (**F**) express the CD52 antigen.

Figure 4: **A-F:** Cells were analyzed for surface (membranous) and cytoplasmic (intracellular) expression of CD52 by flow cytometry. The black curve represent cells stained with the CD52:FITC antibody, the grey curve represents the isotype control staining (IgG:FITC).

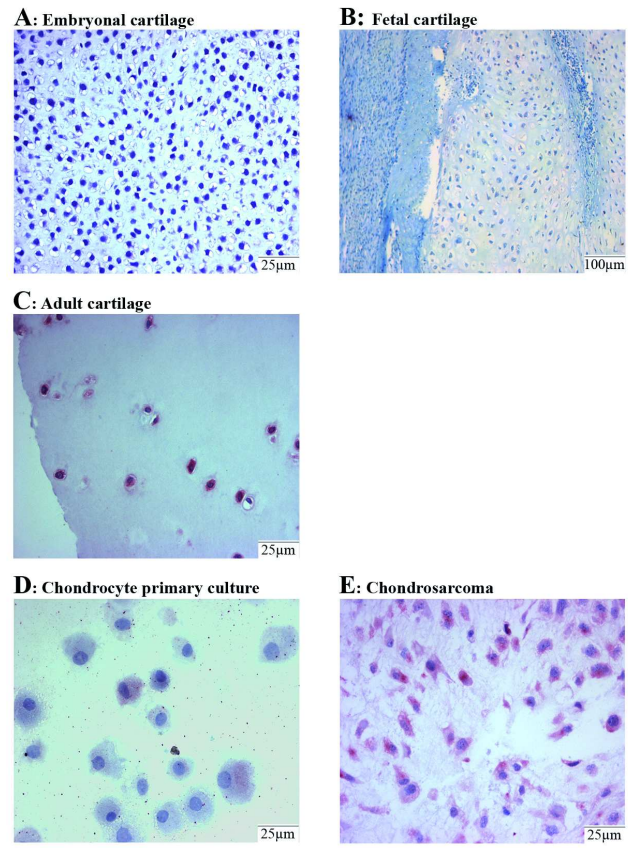
Figure 5: Complement-dependent (A), dose- (B) and time-dependent (C) reduction of cell growth through CAMPATH-1H. **D:** Western blot analysis was performed to detect cleavage of PARP. MNNG/HOS cells were treated with 50µg/ml CAMPATH-1H up to three days, afterwards PARP cleavage was tested.

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Figure 1A-E

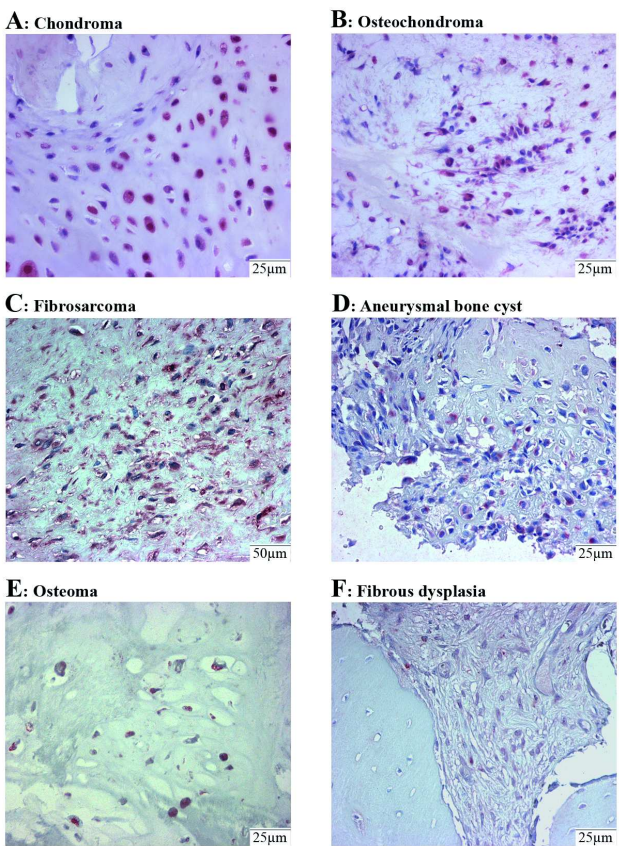


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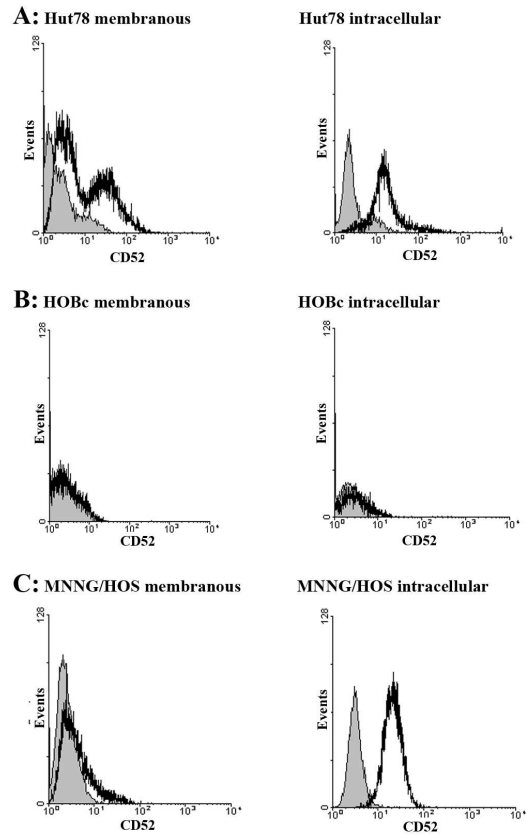
Figure 2A-E

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Figure 3A-F

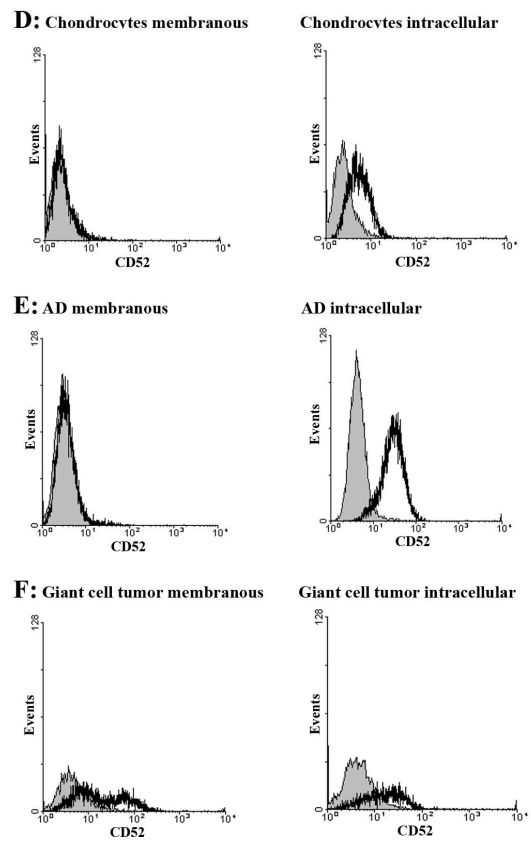


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Figure 4A-C

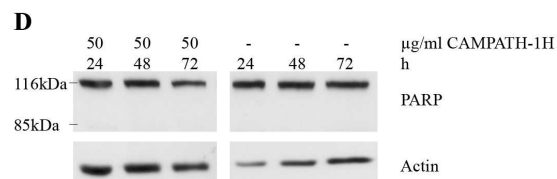
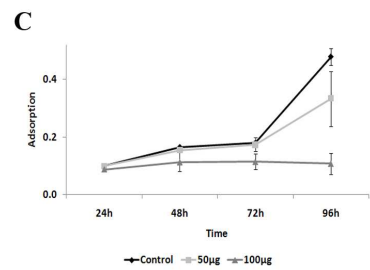
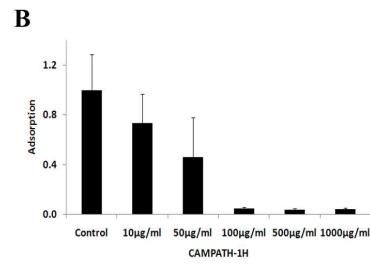
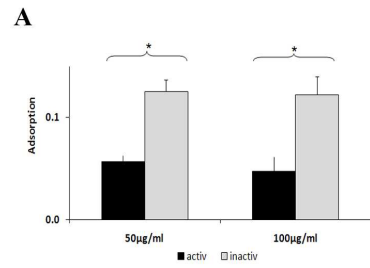
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Figure 4D-F



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Figure 5A-C



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